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Research Report

Filamin-A and Myosin VI colocalize with fibrillary Tau protein in Alzheimer's disease and FTDP-17 brains

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ABSTRACT

Tauopathies, including Alzheimer's disease (AD), fronto-temporal dementia with parkinsonism linked to chromosome 17 (FTDP-17), Pick's disease and progressive supranuclear palsy, are neurodegenerative disorders neuropathologically characterized by the presence of intraneuronal fibrillary inclusions composed of abnormally phosphorylated-Tau. Tau protein is a neuronal microtubule-associated protein (MAP) involved in microtubules polymerization and stabilization. So far, the molecular mechanisms underlying Tau-mediated cellular toxicity remain elusive. To address the determinants of Tau neurotoxicity, we previously performed a misexpression screening in a *Drosophila* tauopathy model to identify genetic modifiers of the human Tau-induced neurodegeneration. We identified several components of the actin network as modifiers of Tau V337M-induced neurodegeneration, i.e. Filamin-A, Myosin VI, Paxillin and Transgelin-3. The aim of this study was to assess whether these genetic interactions were associated with a colocalization of the proteins (i) in the brains of patients with Tau pathologies, and (ii) in the brain of transgenic mice overexpressing human mutant Tau. We found that Filamin-A and Myosin VI indeed colocalize with fibrillary Tau protein in AD and FTDP-17 and in Thy-Tau22 transgenic mice.

1. Introduction

Tau protein is the major neuronal microtubule-associated protein (MAP), predominantly found in axons. In adult human brain, six Tau isoforms, resulting from alternative mRNA splicing (for review, see: Buee et al., 2000; Lee et al., 2001; Sergeant et al., 2005), are encoded by the *MAPT* gene located on chromosome 17q21. This alternative splicing gives rise to isoforms containing three or four microtubule-binding domains, called Tau3R and Tau4R. Physiologically, Tau is a

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developmentally regulated MAP that influences microtubules assembly and stabilization by directly associating with tubulin. Its biological activity is highly regulated by its phosphorylation state. In addition to phosphorylation, the alternative splicing also affects the biological activity of Tau. Indeed, Tau4R isoforms have a greater microtubule-binding affinity than Tau3R isoforms and therefore are more efficient at promoting microtubule assembly.

Tauopathies are neurodegenerative disorders resulting from the alteration of the Tau protein biology. These disorders can be split into primary tauopathies (e.g. FTDP-17), which result from mutations in the *MAPT* gene, and secondary tauopathies including Alzheimer's disease (AD), sporadic Pick's disease (PiD), corticobasal degeneration (CBD), progressive supranuclear palsy (PSP) and argyrophilic grain disease in which aberrant post-translational modifications of Tau are not associated with mutations in *MAPT* gene.

Tauopathies are neuropathologically characterized by the presence of intraneuronal fibrillary inclusions composed of abnormally and hyperphosphorylated Tau. Depending on the pathological isoforms and post-translational modifications of Tau, the filaments that form these inclusions differ ultrastructurally. Indeed, the major components of neurofibrillary tangles (NFTs) found in AD are paired-helical filaments (PHF). In FTDP-17, Tau protein aggregates into twisted ribbon filaments, PHF and straight filaments depending on mutations. Pick bodies consist of both random coiled and straight filaments. Straight filaments are predominant in PSP. Moreover, each of these disorders is characterized by a specific regional and laminar pattern of fibrillary inclusions. In AD, NFTs are preferentially observed in the large pyramidal cells of the hippocampus and the entorhinal cortex, and in the supragranular (II–III) and infragranular (V–VI) layers of the association cortical areas. Many cortical and subcortical areas, such as nucleus basalis of Meynert, amygdala, locus coeruleus and dorsal raphe, are also affected by NFT formation. In FTDP-17, histology showed fibrillary lesions both in cerebral cortex, cerebellum, brainstem and spinal cord. Pick bodies are most numerous in layers II and VI of the neocortex and in the dentate granule neurons of the hippocampus. In PSP, NFTs were described in basal ganglia, brainstem, cerebellum, spinal cord, and in the perirhinal, inferior temporal and prefrontal cortex, with variable densities among cases.

Currently, despite extensive research, molecular mechanisms that lead from the alteration of the Tau biology to neuronal death are still unknown whatever the tauopathy considered. To address the determinants of Tau neurotoxicity, we have previously performed a misexpression screening in a *Drosophila* primary tauopathy model to identify genetic modifiers of the human Tau-induced neurodegeneration (Blard et al., 2007). *Drosophila* has proved to be a powerful model for studying tauopathies, as it recapitulates several relevant phenotypes of these diseases: early death, synaptic dysfunction and neurodegeneration (Chee et al., 2006; Chee et al., 2005; Williams et al., 2000; Wittmann et al., 2001). The screening of 1250 mutant *Drosophila* lines allowed us to identify 30 specific modifiers of Tau V337M-induced neurodegeneration, among which several components of the cytoskeleton, and particularly of the actin network: *i)* Filamin-A, involved in cortical actin filament cross-link (for review, see: Stossel et al., 2001); *ii)* Myosin VI, involved in vesicle and organelle transport along the actin cytoskeleton (for review, see: Cramer, 2000); *iii)* Paxillin, a member of the focal adhesion complex which plays the role of an adaptor between plasma membrane and the actin cytoskeleton (for review, see: Brown and Turner, 2004; Turner, 2000), and *iv)* Transgelin-3, a neuronal protein which colocalizes with microtubules and actin microfilaments (de las Heras et al., 2007; Depaz et al., 2005). Crosstalk between the actin microfilament network and the microtubule cytoskeleton has recently attracted much attention. Fulga et al. found that the overexpression of the FTDP-17 R406W mutant form of Tau causes actin filaments to bundle into rod-shaped aggregates in *Drosophila* and mouse models of tauopathy, and that these actin rearrangements correlate directly with the degree of neurodegeneration (Fulga et al., 2007). They also identified actin as a Tau-interacting protein and as a downstream effector of TauV337M-induced degeneration in *Drosophila*. Altogether, these data suggest that when components of the microtubules and microfilaments networks are dysregulated, their abnormal interaction may have dramatic consequences for the neurons, potentially leading to neuronal death observed in tauopathies. This study was performed to investigate whether the genetic interactions identified in *Drosophila* also implicated a colocalization of these brain proteins in brains of patients with different tauopathies and in transgenic mice overexpressing human Tau mutated at sites G272V and P301S (Thy-Tau22 mice).

2. Results

To assess the relevance of the genetic interactions we previously identified in a *Drosophila* tauopathy model towards the human pathologies, we studied by immunohistochemistry the sub-cellular location of Filamin-A, Myosin VI, Paxillin and Transgelin-3 in the brain of Thy-Tau22 transgenic mice (Schindowski et al., 2006) and in the brains from patients with AD, FTDP-17, PiD, or PSP. For each pathological condition, we decided to study only regions in which the Tau fibrillary lesions are numerous (Table 1).

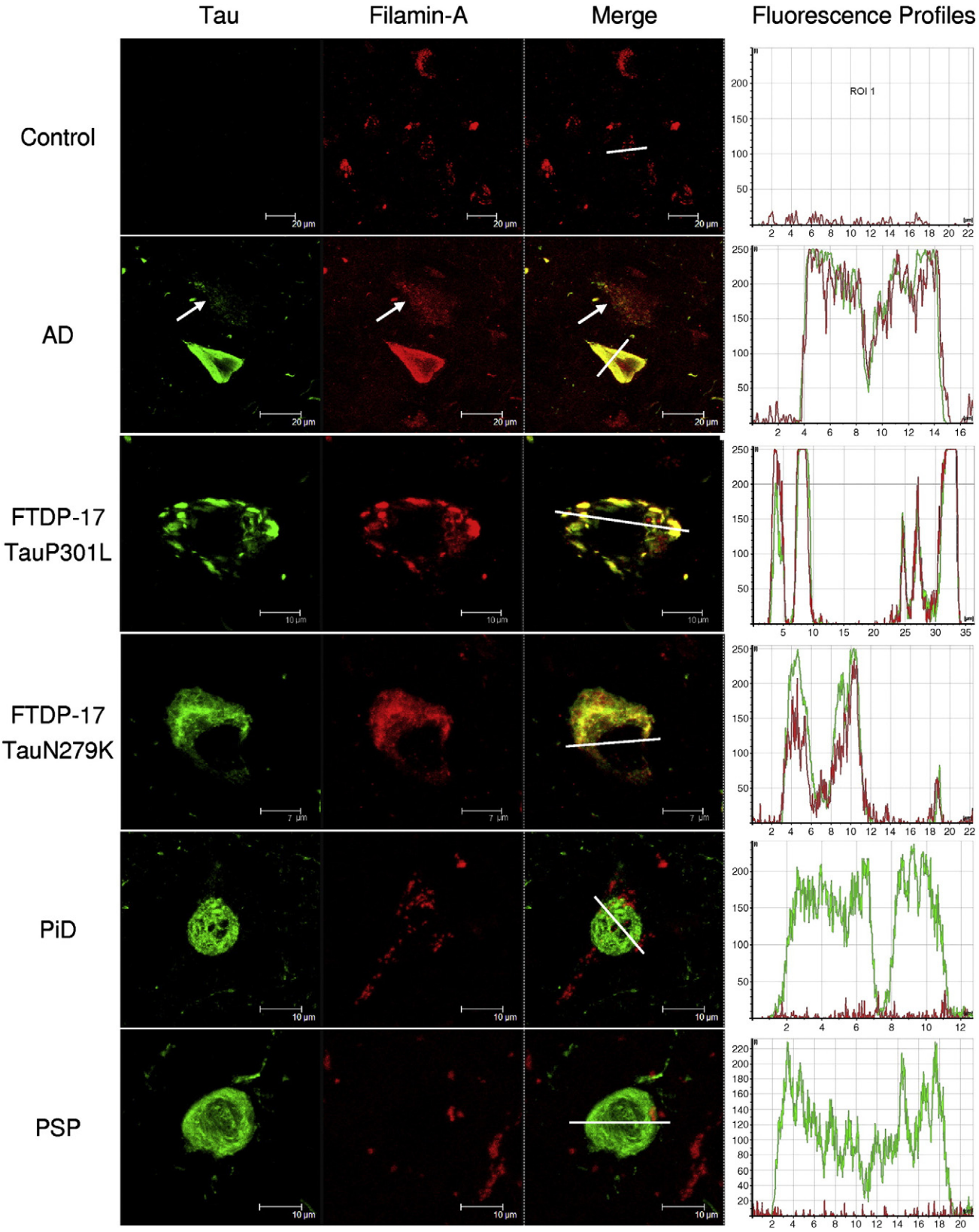
Filamin-A signal was low in the hippocampus and frontal cortex from controls, except in astrocytes which are slightly labeled and in blood vessels (data not shown). Arteries, capillaries and veins in parenchyma as well as in the meninges were strongly labeled. In the hippocampus and frontal cortex

Table 1 – This table lists the cases and brain's areas studied.

Case	Mutations	Age (y)	Sex	Postmortem delay (h)	Brain's areas
Control 1	/	52	Male	13	H, FC
Control 2	/	60	Male	6	H, FC
AD 1	/	62	Male	10	H, FC
AD 2	/	78	Male	48	H, FC
FTDP-17 1	Tau P301L	66	Female	24	H, FC
FTDP-17 2	Tau P301L	65	Female	31	H, FC
FTDP-17 3	Tau N279K	47	Male	un	H, FC
PiD	/	57	Male	22	H
PSP	/	57	Male	20	M

H: hippocampus, FC: frontal cortex, M: mesencephalon, un: unknown.

from the two AD patients, NFTs, neuropil threads and neuritic plaques were strongly positive (Figs. 1 and 2). In FTDP-17, whatever the *MAPT* mutation considered, and whatever the patient considered for the same mutation, Filamin-A-labeling was observed in NFTs, glial fibrillary tangles (GFTs) and coiled bodies in the hippocampus and frontal cortex (Figs. 1 and 2). In both conditions, the "colocalization profile" of Filamin-A and Tau confirmed the high degree of colocalization of the two



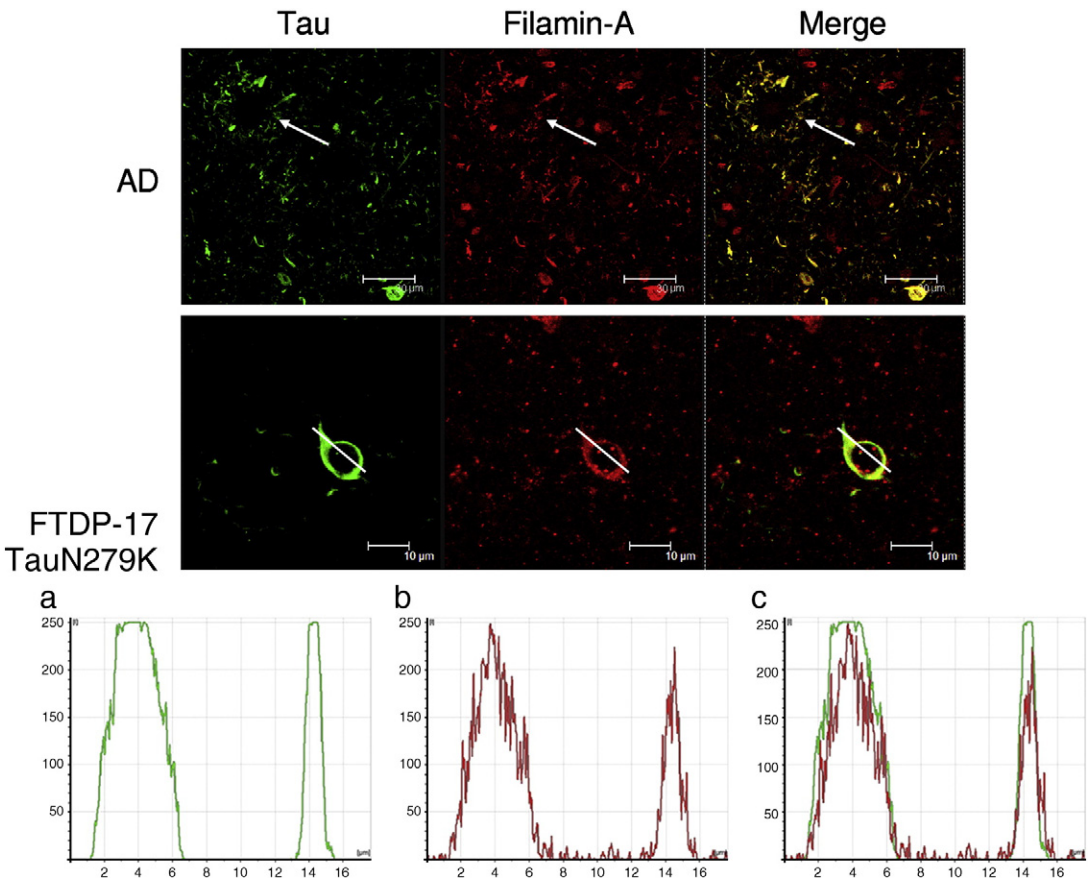


Fig. 2 – Colocalization of Filamin-A and Tau in neuropil threads and dystrophic neurites in AD brains and in GFTs in FTDP-17 brains. Confocal images showing double-stained brain sections from AD and FTDP-17 TauN279K patients (AD: hippocampus, temporal neocortex; FTDP-17 TauN279K: frontal cortex). In green, anti-Tau labeling. In red, anti-Filamin-A labeling. In (a), (b) and (c), fluorescence profiles. Neuropil threads and dystrophic neurites (arrow) in AD brains and GFTs in FTDP-17 brain were also Filamin-A-positive.

proteins. This colocalization was observed whatever the fibrillarization state considered, from pre-tangle to ghost-tangle. In contrast, in PiD and PSP patients, no Filamin-A-labeling was detectable in Tau-positive cells (Fig. 1). In particular, neither Pick bodies in the hippocampus from the PiD case nor NFTs in the mesencephalon from the PSP case were labeled. Altogether, these observations show that Filamin-A colocalizes with fibrillary Tau protein in AD and FTDP-17. Moreover, its accumulation is not a common feature of all the tauopathies. Note that a colocalization of Filamin-A and Tau in NFTs was also found in the hippocampus from the Thy-Tau22 mice (Fig. S1).

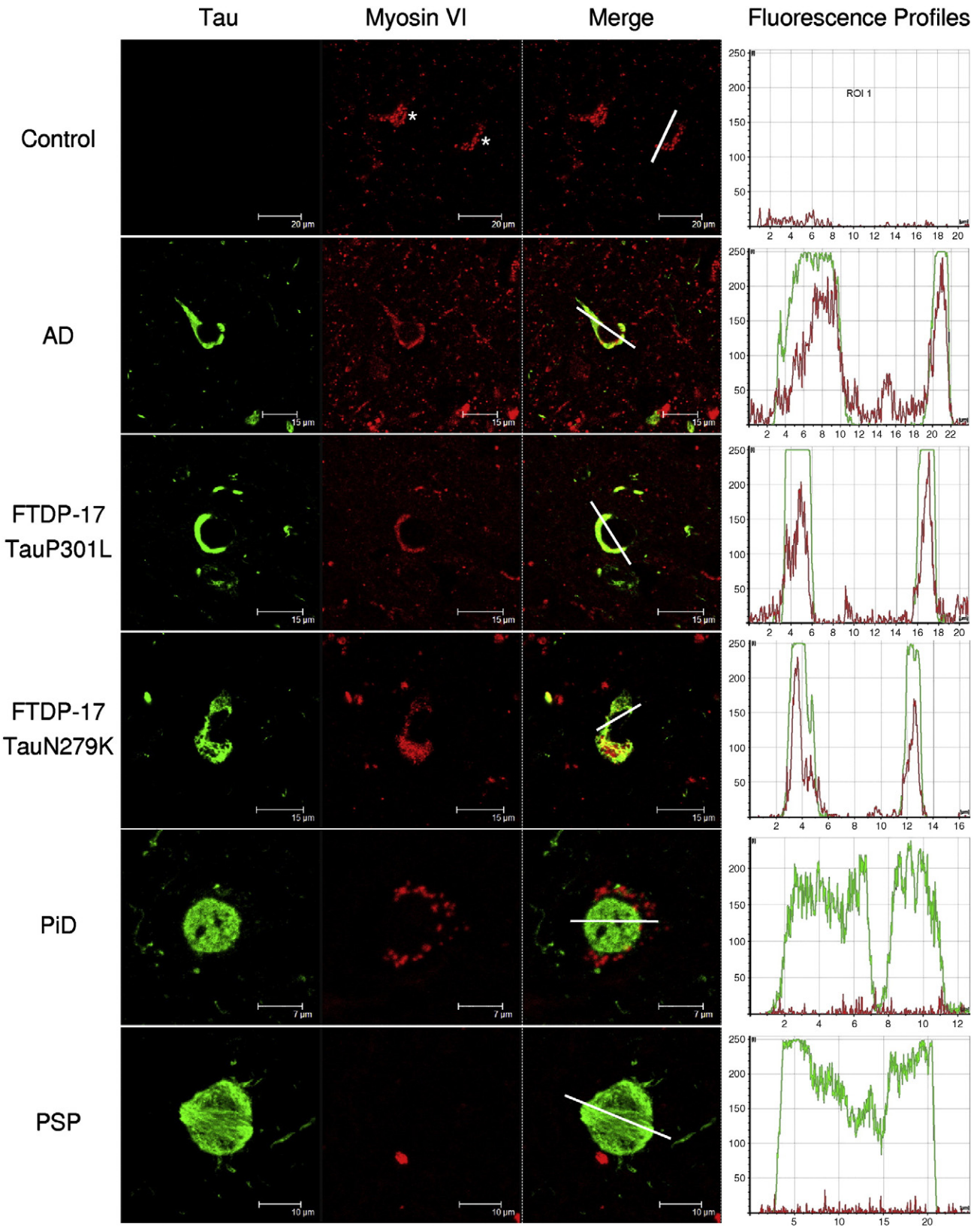
Myosin VI immunostaining was strong in neuropil and low in white matter in the hippocampus and the frontal cortex from the control brains (data not shown). Moreover, the Myosin VI antibody labeled the mature and ghost tangles in the two AD and the three FTDP-17 patients. As previously, the "co-localization profile" confirmed the colocalization of Myosin VI and Tau in these fibrillary lesions. At the opposite, Pick bodies and PSP tangles were Myosin VI-negative (Fig. 3), showing that, as for the Filamin-A, Myosin VI colocalizes with Tau only in AD and FTDP-17 among the four tauopathies considered. The Thy-Tau22 mice also displayed Myosin VI and Tau colocalization (Fig. S2).

The two Paxillin antibodies we used diffusely and weakly labeled the neuropil while the white matter was negative in the hippocampus and frontal cortex from controls as well as from the AD, FTDP-17, PiD and PSP cases. The NFTs and Pick

Fig. 1 – Colocalization of Filamin-A and Tau in NFTs in AD and FTDP-17 brains. Confocal images showing double-stained brain sections from control (hippocampus, entorhinal cortex) and patients with Tau pathologies (AD: hippocampal CA1 region; FTDP-17 TauP301L: hippocampus, entorhinal cortex; FTDP-17 TauN279K: hippocampal CA4 region; PiD: hippocampal region CA2; PSP: mesencephalon). In green, anti-Tau labeling. In red, anti-Filamin-A labeling. In controls, Filamin-A-labeling was diffuse and, as expected, no pathological Tau labeling was observed. The red staining corresponded to autofluorescent lipofuscin grains. In AD and FTDP-17, the NFTs, labeled with the anti-Tau AT8 antibody, were Filamin-A-positive. The fluorescence profiles confirmed the colocalization of the two proteins. The arrow in the AD brain section points a neurofibrillary tangle which was being formed. In contrast, the Pick bodies and the NFTs from the PSP case were Filamin-A-negative.

bodies in brains from patients were Paxillin-negative (Fig. S3), indicating that Paxillin does not colocalize with the fibrillary Tau protein in human. Altogether, these data suggest that the sub-cellular location of Paxillin is not altered in tauopathies.

Transgelin 3 slightly immunopositive cells were present in the cortex as well as in the hippocampal CA3 and CA4 regions from the control brains. No change in Transgelin-3 labeling was observed in patients, showing that Transgelin-3 does not accumulate in Tau-positive cells (Fig. S4).



3. Discussion

Two of the four modulators of Tau pathology identified in *Drosophila*, Filamin-A and Myosin VI, colocalized with Tau protein accumulated in neurons of AD cases and in neurons and glia of FTDP-17 cases with the P301L and N279K mutations. Among the four proteins included in this study, two, Paxillin and Filamin-A, had previously been characterized in the brain of patients. In AD brains, Grace and Busciglio have shown a strong immunolabeling of senile plaques, dystrophic neurites and cell bodies surrounding the plaques core by phosphorylated (activated) Paxillin (Grace and Busciglio, 2003). We could not reproduce this result in our AD cases with two different antibodies, one of them being the same clone as the one used by these authors. We have no explanation for this discrepancy. Zhang et al. have previously detected a robust Filamin-A immunostaining in cortical and hippocampal CA1 intraneuronal NFTs and neuropil threads as well as in dystrophic neurites of AD brains (Zhang et al., 1998). In this study, the immunochemistry was done on adjacent sections. Our study shows that Filamin-A and Tau colocalize and extends these data to FTDP-17. Finally, Myosin VI and Transgelin-3 had never been implicated before in neurodegenerative diseases. Moreover, no neuropathological data were available for Myosin VI in human. In the present study, we show for the first time that Myosin VI colocalizes with fibrillary Tau protein in AD and FTDP-17.

We next investigated whether the genetic interactions identified in *Drosophila* also implicated a colocalization of these brain proteins in Thy-Tau22 transgenic mice. Filamin-A and Myosin VI colocalize with pathological Tau in hippocampus and in hippocampus and amygdala from these mice respectively. Note that the labelings of Paxillin and Transgelin-3 were inconclusive.

The ability of Filamin-A and Myosin VI to modulate the Tau-induced toxicity in flies, and the colocalization of these two modulators with Tau in neurofibrillary tangles in AD and FTDP-17 raise the possibility that they play a role in the neurodegenerative process. These data also imply that a new dividing line may be drawn among tauopathies separating those with and those without accumulation of Filamin-A and Myosin VI. Our findings raise several questions: *i*) What is the consequence of the accumulation of these two components of the actin network on cell functioning and survival? *ii*) Why don't Filamin-A and Myosin VI accumulate in PiD and PSP brains?

Filamin-A protein has been found to promote branching of actin filaments and is thought to modulate cell shape, polarity, and motility via changes in actin filament organization. Filamentous actin (F-actin) is the major cytoskeletal components of synaptic terminals and is highly involved in the maintenance of the synaptic plasticity (for review, see: Cingolani and Goda, 2008). It could therefore be hypothetized that Tau-induced depletion of Filamin-A is a key event leading to destabilization of the actin network and to synaptic loss. Myosin VI is a protein enriched in postsynaptic compartments of glutamatergic synapses and involved in vesicle and organelle transport along the actin cytoskeleton. Osterweil et al. have shown in a murine model that loss of Myosin VI leads to a decrease in synaptic density and to dendritic spine abnormalities in whole brain and isolated hippocampal neurons. Similarly, hippocampal neurons expressing dominant-negative Myosin VI exhibit a decrease in synapse number, which suggests that acute Myosin VI disruption at mature synapses results in their elimination (Osterweil et al., 2005). Moreover, Nash et al. have shown in a rat model that the disruption of the interaction between Myosin VI and SAP97 (synapse associated protein 97) is associated with a reduction in the number of alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate-type glutamate receptors (AMPARs) at hippocampal synapses (Nash et al., 2010). In our study, we found that Myosin VI colocalization with fibrillary Tau protein was restricted to mature and ghost tangles, suggesting that its accumulation does not constitute one of the initial events of the neurodegenerative process in AD and FTDP-17 but could participate to synaptic dysfunction leading to neuronal death by disrupting organelle and vesicle actin-based transport at terminal nerves. Interestingly, we had previously identified several components involved in synaptic plasticity maintenance as modulators of Tau-induced toxicity in *Drosophila* (Blard et al., 2007).

Tau isoforms that compose intraneuronal inclusions differ with tauopathies: Pick bodies are composed of Tau3R whereas PSP filaments are composed of Tau4R. In FTDP-17, TauN279K and TauP301L mutations lead to the accumulation of Tau4R because the first enhances the inclusion of the exon 10 coding the second microtubules binding domain of Tau whereas the second reduces the ability of 4R-Tau isoforms to interact with microtubules. In AD, tangles are made of both 3R and 4R-Tau. Consequently, our results show that the accumulation of Filamin-A and Myosin VI is independent of the type of Tau isoforms found in lesions. It also seems to be independent of the brain region considered. Indeed, for a same region (hippocampus), Filamin-A and Myosin VI accumulate in AD and FTDP-17 but not in PiD.

Interestingly, previous molecular and immunohistochemical experiments demonstrated that NFTs contain a number of cytoskeletal proteins, including neurofilament proteins, vimentin, actin, and cofilin (Wang et al., 2005). In this study, we identified in NFTs two new actin-binding proteins, Filamin-A and Myosin VI. In conclusion, these data support the view that

Fig. 3 – Colocalization of Myosin VI and pathological Tau in AD and FTDP-17 brains. Confocal images showing double-stained brain sections with Tau and Myosin VI antibodies from control (frontal cortex) and patients with Tau pathologies (AD: frontal cortex; FTDP-17 TauP301L: hippocampus, dentate gyrus; FTDP-17 TauN279K: hippocampal region CA4; PiD: hippocampal region CA3; PSP: mesencephalon). In green, anti-Tau labeling. In red, anti-Myosin VI-labeling. In control brain, neurons were weakly stained with the anti-Myosin VI antibody. The asterisks point autofluorescent lipofuscin grains. In the AD and FTDP-17 brains, the NFTs were strongly stained with the anti-Myosin VI antibody. In addition, the accumulated Tau and Myosin VI proteins colocalized, as confirmed by the fluorescence profiles. In contrast, Pick bodies and NFTs in the brain of the PSP case were Myosin VI-negative.

Table 2 – Primary antibodies used for immunohistochemistry on human tissues.

Antigen/clone	Poly/monoclonal	Immunogen	Producers	Dilution
Tau (AT8)	Monoclonal (mouse)	Partially purified human PHF-tau	Innogenetics (Gent, Belgium)	1:20
Tau	Polyclonal (rabbit)	Recombinant human tau protein corresponding to the C-terminal part (amino acids 243–441) containing the four repeated sequences involved in microtubule binding.	Dako (Glostrup, Denmark)	1:200
Filamin-A (H-300)	Polyclonal (rabbit)	Amino acids 2348–2647 mapping at the C-terminus of Filamin-A of human origin	Santa Cruz Biotechnology (Santa Cruz, USA)	1:10
Myosin VI (H-215)	Polyclonal (rabbit)	Amino acids 1071–1285 mapping at the C-terminus of Myosin VI of human origin	Santa Cruz Biotechnology (Santa Cruz, USA)	1:10
Paxillin pY^{31}	Polyclonal (rabbit)	Chemically synthesized phosphopeptide derived from the region of human Paxillin that contains tyrosine 31.	Invitrogen (Carlsbad, USA)	1:20
Paxillin	Monoclonal (mouse)	Chicken Paxillin — amino acids 1–557	BD Biosciences (Erembodegem, Belgium)	1:200
Transgelin-3 (438.1)	Monoclonal (mouse)	Recombinant human Transgelin-3	Santa Cruz Biotechnology (Santa Cruz, USA)	1:100

the Tau-induced toxicity in AD and FTDP-17 could be mediated by disruption of the actin network, leading to synaptic loss and neuronal death that occur in these two diseases.

4. Experimental procedures

4.1. *Human study*

4.1.1. *Cases*

Two sporadic AD cases (62 and 78 years), three FTDP-17 cases (47, 65 and 66 years), one sporadic PiD case (57 years), one sporadic PSP case (57 year) as well as two control subjects without history of dementia or of other neurological disease (52 and 60 years) were studied. The identified mutations in the FTDP-17 cases were TauP301L (two cases) and TauN279K (one case). Age, sex, and post mortem delay are summarized in Table 1.

4.1.2. *Brain tissue*

All cases had signed informed consent for autopsy and the conservation of post mortem samples (consent for research) according to French law. For each case, the final neuropathological diagnosis was made by trained neuropathologists according to current criteria. Depending on each case, samples from hippocampus, frontal cortex or mesencephalon, fixed first in 10% buffered formalin, were embedded in paraffin. For each sample, sections of 7 μm thick were obtained (Table 1).

4.1.3. *Immunohistochemistry/immunofluorescence*

After deparaffinization and rehydration, sections were pretreated by heat for 40 min in citrate buffer (10 mM) pH 6 at 95 °C to unmask the epitopes. After incubation for 1 h in phosphate buffer saline (PBS) containing triton 0.2% and bovine serum albumin (BSA) 0.5% in order to limit unspecific fixation of the antibodies, sections were co-immunolabeled for 1 h at room temperature with an anti-Tau, human specific, antibody and with an antibody that recognizes Filamin-A, Myosin VI, Paxillin or Transgelin-3. Primary antibodies used for immunohistochemistry are described in Table 2.

The labeling was visualized by immunofluorescence with secondary antibodies coupled either with the Alexa 488 fluor dye (Molecular probes, Eugene, OR, USA) which fluoresces green, or with the Alexa 594 fluor dye (Molecular probes) which fluoresces red. Between the steps, sections were rinsed in PBS containing BSA 0.5%. The immunofluorescence was analysed with a Leica laser-scanning confocal microscope (TCS-SP2). To confirm the distribution of the interactors in the Tau-positive cells, we measured the intensity of the transmitted light in the green and the red channels along a line segment drawn across the cells of interest. This measurement of "colocalization profile" was performed on a single stack of each confocal image using the Leica confocal Lite software v2.61.

4.2. *Murine study*

THY-Tau22 male mice that express the 412 amino acid 4-repeat isoform of human tau mutated at sites G272V and P301S under a Thy1.2-promotor, aged of 9 months, were used in the present study. THY-Tau22 mice were bred with C57Bl6 females and the progeny was genotyped using PCR on DNA isolated from tail biopsy as described previously (Belarbi et al., 2009; Schindowski et al., 2006). Animals were sacrificed and brain removed. Brains were fixed for 7 days in 4% paraformaldehyde, then incubated in 20% sucrose for 24 h and finally kept frozen until use. Free-

Table 3 – Primary antibodies used for immunohistochemistry on murine tissues.

Antigen/clone	Poly/monoclonal	Immunogen	Producers	Dilution
Tau (AT8)	Monoclonal (mouse)	Partially purified human PHF-tau	Pierce (Rockford, USA)	1:200
Filamin-A (H-300)	Polyclonal (rabbit)	Amino acids 2348–2647 mapping at the C-terminus of Filamin-A of human origin	Santa Cruz Biotechnology (Carlsbad, USA)	1:200
Myosin VI (H-215)	Polyclonal (rabbit)	Amino acids 1071–1285 mapping at the C-terminus of Myosin VI of human origin	Santa Cruz Biotechnology (Carlsbad, USA)	1:200

floating coronal sections (40 μm) were obtained using a cryostat (Leica). Sections of interest were used for free-floating immunohistochemistry as previously described (Belarbi et al., 2009) and finally mounted on Superfrost slides respectively. Primary antibodies used for immunohistochemistry are described in Table 3. The immunofluorescent labelings were analysed with a Zeiss ApoTome fluorescence microscope.

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The authors declare that they have no conflicts of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.brainres.2010.05.007.

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